

APPENDIX A

(CLEAN VERSION OF SUBSTITUTE SPECIFICATION)

(Serial No. 09/679,664)



G-PROTEIN FUSION RECEPTORS AND CONSTRUCTS ENCODING SAME

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] The present application is a continuation-in-part of PCT/US99/07333, which claims priority to Garrett *et al.* U.S. Serial No. 60/080,671, filed April 3, 1998, which is hereby incorporated by reference herein in its entirety including the drawings.

FIELD OF THE INVENTION

[0002] The present invention relates to G-protein fusion receptors, chimeric GABA_B (γ -aminobutyric acid) receptors, nucleic acid encoding such receptors, and uses of such receptors, and nucleic acid encoding such receptors.

BACKGROUND

[0003] The references cited herein are not admitted to be prior art to the claimed invention.

[0004] Chimeric receptors made up of peptide segments from different receptors have different uses, such as being used to assess the functions of different sequence regions and to assess the activity of different compounds at a particular receptor. Examples of using chimeric receptors to assess the activity of different compounds are provided by Dull *et al.*, U.S. Patent No. 4,859,609; Dull *et al.*, U.S. Patent No. 5,030,576; Fuller *et al.*, U.S. Patent No. 5,981,195; and International Application No. PCT/US96/12336, International Publication No. WO 97/05252.

[0005] Dull *et al.* U.S. Patent No. 4,859,609, and Dull *et al.* U.S. Patent No. 5,030,576, indicate the production and use of chimeric receptors comprising a ligand-binding domain of a predetermined receptor and a heterologous reporter polypeptide. The Dull *et al.* patents provide as examples of chimerics: (1) a chimeric receptor made up of the insulin receptor extracellular α chain, and the EGF receptor transmembrane and cytoplasmic domains without any HIR B-chain sequence; and (2) a hybrid receptor made up of the v-erbB oncogene product intracellular domain fused to the EGF receptor extracellular and transmembrane domains.

[0006] Fuller *et al.* International Publication No. WO 97/05252 feature chimeric receptors made up of metabotropic glutamate receptor (mGluR) domains and calcium receptor (CaR) domains. The chimeric receptors allow the coupling of functional aspects of a mGluR with a CaR.

[0007] An example of the use of chimeric receptors to assess the functions of different sequence region receptors are found in studies identifying regions of different guanine nucleotide-binding protein-coupled receptors important for guanine nucleotide-binding protein coupling. (See, Kobilka *et al.*, *Science* 240:1310-1316, 1988; Wess *et al.*, *FEBS Lett.* 258:133-136, 1989; Cotecchia *et al.*, *Proc. Natl. Acad. Sci. USA* 87:2896-2900, 1990; Lechleiter *et al.*, *EMBO J.* 9:4381-4390, 1990; Wess *et al.*, *Mol. Pharmacol.* 38:517-523, 1990; and Pin *et al.*, *EMBO J.* 13:342-348, 1994.)

SUMMARY OF THE INVENTION

[0008] The present invention features G-protein fusion receptors and chimeric GABA_B receptors (GABA_BRs), nucleic acid encoding such receptors, and the use of such receptors and nucleic acid. G-protein fusion receptors comprise at least one domain from a CaR, a mGluR, and/or a GABA_B receptor fused directly or through a linker to a guanine nucleotide-binding protein (G-protein). Chimeric GABA_BRs comprise at least one of a GABA_BR extracellular domain, a GABA_BR transmembrane domain, or a GABA_BR intracellular domain and one or more domains from a mGluR subtype 8 (mGluR8) and/or a CaR.

[0009] G-proteins are peripheral membrane proteins made up of an α subunit, a β subunit, and a γ subunit. G-proteins interconvert between a GDP-bound and a GTP-bound form. Different types of G-proteins can affect different enzymes, such as adenylate cyclase and phospholipase-C.

[0010] Thus, a first aspect of the present invention describes a G-protein fusion receptor comprising:

[0011] an extracellular domain comprising an extracellular domain amino acid sequence substantially similar to either an extracellular CaR amino acid sequence, an extracellular mGluR amino acid sequence, or an extracellular GABA_B receptor amino acid sequence;

[0012] a transmembrane domain joined to the carboxy terminus of the extracellular domain, the transmembrane domain comprising a transmembrane domain amino acid sequence substantially similar to either a transmembrane CaR amino acid sequence, a transmembrane mGluR amino acid sequence, or a transmembrane GABA_B receptor amino acid sequence;

[0013] an intracellular domain joined to the carboxy terminus of the transmembrane domain comprising all or a portion of an intracellular amino acid sequence substantially similar to either an intracellular CaR amino acid sequence, an intracellular mGluR amino acid sequence, or an intracellular GABA_B receptor amino acid sequence, provided that the portion is at least about ten amino acids;

[0014] an optionally present linker joined to the carboxy terminus of the intracellular domain, where the optionally present linker is a polypeptide 3 to 30 amino acids in length, wherein the amino acids of the optionally present linker are selected from the group consisting of alanine, proline, serine, and glycine; and

[0015] a G-protein joined either to the intracellular domain or to the optionally present linker, provided that the G-protein is joined to the optionally present linker when the optionally present linker is present.

[0016] “Substantially similar” refers to at least 40% sequence similarity between respective polypeptide regions making up a domain. In preferred embodiments, “substantially similar” refers to at least 50%, at least 75%, at least 90%, at least 95% sequence similarity, or 100% (the same sequence), between polypeptide domains. The degree to which two polypeptide domains are substantially similar is determined by comparing the amino acid sequences located in corresponding domains. Sequence similarity is preferably determined using BLASTN (Altschul *et al.*, *J. Mol. Biol.* 215:403-410, 1990).

[0017] The different receptor components of the G-protein receptor can come from the same receptor protein or from a chimeric receptor made up of different receptor domains. By swapping different domains, compounds able to effect different domains of a particular receptor can be identified and the activity of different compounds at different domains can be measured.

[0018] Different embodiments the CaR region(s) present in the G-protein fusion are substantially similar to, comprise, or consist of portion(s) of a mammalian CaR, preferably, the human CaR; mGluR region(s) present in the G-protein fusion are substantially similar to,

comprise, or consist of portion(s) of a mammalian mGluR, preferably a human mGluR; and GABA_BR region(s) present in the G-protein fusion are substantially similar to, comprise, or consist of portion(s) of a mammalian GABA_BR, preferably a human GABA_BR receptor.

[0019] In preferred embodiments concerning GABA_BR regions that are present: the GABA_BR extracellular domain is substantially similar to a GABA_BR extracellular domain provided in SEQ. ID. NOs. 2-4; the GABA_BR transmembrane domain is substantially similar to the GABA_BR transmembrane domain provided in SEQ. ID. NOs. 7-9; and the GABA_BR intracellular domain is substantially similar to a GABA_BR intracellular domain provided in SEQ. ID. NOs. 12-14.

[0020] In preferred embodiments concerning CaR regions that are present: the CaR extracellular domain is substantially similar to the CaR extracellular domain provided in SEQ. ID. NO. 1; the CaR transmembrane domain is substantially similar to the CaR transmembrane domain provided in SEQ. ID. NO. 6; and the CaR intracellular domain is substantially similar to the CaR intracellular domain such as that provided in SEQ. ID. NO. 11.

[0021] Various different mGluR subtypes present in different organisms, including humans, are described in different patent publications as follows: mGluR₁ - WO 94/29449, EP 569 240 A1, WO 92/10583 and U.S. Patent No. 5,385,831; mGluR₂ - WO 94/29449, WO 96/06167, and EP 711 832 A2; mGluR₃ - WO 94/29449, and WO 95/22609; mGluR₄ - WO 95/08627, WO 95/22609, and WO 96/29404; mGluR₅ - WO 94/29449; mGluR₆ - WO 95/08627; mGluR₇ - U.S. Patent No. 5,831,047, WO 95/08627 and WO 96/29404; and mGluR₈ - U.S. Patent Nos. 6,051,688, 6,077,675, 6,084,084, WO 97/48724 and EP 816 498 A2. (Each of these references is hereby incorporated by reference herein.)

[0022] In preferred embodiments concerning mGluR regions that are present: the mGluR extracellular domain is substantially similar to either human mGluR 1, human mGluR 2, human mGluR 3, human mGluR 4, human mGluR 5, human mGluR 6, human mGluR 7, or human mGluR 8; the mGluR transmembrane domain is substantially similar to either human mGluR 1, human mGluR 2, human mGluR 3, human mGluR 4, human mGluR 5, human mGluR 6, human mGluR 7, or human mGluR 8; and the mGluR intracellular domain is substantially similar to either human mGluR 1, human mGluR 2, human mGluR 3, human

mGluR 4, human mGluR 5, human mGluR 6, human mGluR 7, or human mGluR 8. Preferred embodiments also include any mGluR splice variant.

[0023] In preferred embodiments concerning the optionally present linker, the optionally present linker is a polypeptide 3 to 30 amino acids in length, wherein the amino acids of the optionally present linker are selected from the group consisting of alanine, proline, serine, and glycine; and more preferably, the optionally present linker is comprised of alanine amino acids.

[0024] Another aspect of the present invention describes a recombinant cell comprising an expression vector encoding for a G-protein fusion receptor, and a cell where the G-protein fusion receptor is expressed. Preferably, the G-protein fusion receptor is functional in the cell.

[0025] Another aspect of the present invention describes a recombinant cell produced by combining (a) a cell where a G-protein fusion receptor is expressed, and (b) a vector comprising nucleic acid encoding a G-protein fusion receptor and elements for introducing heterologous nucleic acid into the cell. Preferably, the G-protein fusion receptor is functional in the cell.

[0026] Another aspect of the present invention describes a process for the production of a G-protein fusion receptor. The process is performed by growing host cells comprising a G-protein fusion receptor.

[0027] Another aspect of the present invention describes a method of measuring the ability of a compound to affect G-protein fusion receptor activity.

[0028] Another aspect of the present invention describes a chimeric GABA_BR comprising an extracellular domain, a transmembrane domain and an intracellular domain, wherein at least one domain is from a GABA_BR and at least one domain is from CaR or mGluR8. The extracellular domain comprises an amino acid sequence substantially similar to a CaR extracellular domain (SEQ. ID. NO. 1), a GABA_BR1a extracellular domain (SEQ. ID. NO. 2), a GABA_BR1b extracellular domain (SEQ. ID. NO. 3), a GABA_BR2 extracellular domain (SEQ. ID. NO. 4), or a mGluR8 extracellular domain (SEQ. ID. NO. 5).

[0029] The transmembrane domain comprises an amino acid sequence substantially similar to a CaR transmembrane domain (SEQ. ID. NO. 6), a GABA_BR1a transmembrane domain (SEQ. ID. NO. 7), a GABA_BR1b transmembrane domain (SEQ. ID. NO. 8), a GABA_BR2

transmembrane domain (SEQ. ID. NO. 9), or a mGluR8 transmembrane domain (SEQ. ID. NO. 10).

[0030] The intracellular domain comprises an amino acid sequence substantially similar to a CaR intracellular domain (SEQ. ID. NO. 11), a GABA_BR1a intracellular domain (SEQ. ID. NO. 12), a GABA_BR1b intracellular domain (SEQ. ID. NO. 13), a GABA_BR2 intracellular domain (SEQ. ID. NO. 14), or a mGluR8 intracellular domain (SEQ. ID. NO. 15).

[0031] Preferred chimeric GABA_BRs contain at least one mGluR8 intracellular, transmembrane or extracellular domain, or at least one CaR intracellular, transmembrane or extracellular domain. More preferably, the chimeric GABA_BR contains at least one CaR domain.

[0032] In preferred embodiments concerning mGluR8 regions that are present: the mGluR8 extracellular domain is substantially similar to the mGluR8 extracellular domain provided in SEQ. ID. NO. 5; the mGluR8 transmembrane domain is substantially similar to the mGluR8 transmembrane domain provided in SEQ. ID. NO. 10; and the mGluR8 intracellular domain is substantially similar to the mGluR8 receptor intracellular domain provided in SEQ. ID. NO. 15.

[0033] Preferably, the domains are functionally coupled such that a signal from the binding of an extracellular ligand is transduced to the intracellular domain when the chimeric receptor is present in a suitable host cell. A suitable host cell contains the elements for functional signal transduction for receptors coupled to a G-protein.

[0034] Another aspect of the present invention describes a nucleic acid comprising a nucleotide sequence encoding for a chimeric GABA_BR.

[0035] Another aspect of the present invention describes a recombinant cell comprising an expression vector encoding for a chimeric GABA_BR, and a cell where the chimeric GABA_BR is expressed. Preferably, the chimeric GABA_BR is functional in the cell.

[0036] Another aspect of the present invention describes a recombinant cell produced by combining (a) a cell where a chimeric GABA_BR is expressed, and (b) a vector comprising nucleic acid encoding the chimeric GABA_BR and elements for introducing heterologous nucleic acid into the cell. Preferably, the chimeric GABA_BR is functional in the cell.

[0037] Another aspect of the present invention describes a process for the production of a chimeric receptor. The process is performed by growing host cells comprising a chimeric GABA_BR.

[0038] Another aspect of the present invention describes a method of measuring the ability of a compound to affect GABA_BR or mGluR activity. The method is performed by measuring the ability of a compound to affect chimeric GABA_BR or mGluR activity.

[0039] Another aspect of the present invention describes a fusion receptor polypeptide comprising a receptor and a G-protein α subunit, wherein the G-protein α subunit is fused to the intracellular domain of the receptor, provided that the receptor is not an adrenoreceptor.

[0040] Various examples are described herein. These examples are not intended in any way to limit the claimed invention.

[0041] Other features and advantages of the invention will be apparent from the following drawings, the description of the invention, the examples, and the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0042] FIGS. 1a-1d illustrate the amino acid sequences of a human CaR extracellular domain (SEQ. ID. NO. 1), a human GABA_BR1a extracellular domain (SEQ. ID. NO. 2), a human GABA_BR1b extracellular domain (SEQ. ID. NO. 3), a human GABA_BR2 extracellular domain (SEQ. ID. NO. 4), and a human mGluR8 extracellular domain (SEQ. ID. NO. 5).

[0043] FIGS. 2a-2b illustrate the amino acid sequences of a human CaR transmembrane domain (SEQ. ID. NO. 6), a human GABA_BR1a transmembrane domain (SEQ. ID. NO. 7), a human GABA_BR1b transmembrane domain (SEQ. ID. NO. 8), a human GABA_BR2 transmembrane domain (SEQ. ID. NO. 9), and a human mGluR8 transmembrane domain (SEQ. ID. NO. 10).

[0044] FIGS. 3a-3b illustrate the amino acid sequences of a human CaR intracellular domain (SEQ. ID. NO. 11), a human GABA_BR1a intracellular domain (SEQ. ID. NO. 12), a human GABA_BR1b intracellular domain (SEQ. ID. NO. 13), a human GABA_BR2 intracellular domain (SEQ. ID. NO. 14), and a human mGluR8 intracellular domain (SEQ. ID. NO. 15).

[0045] FIGS. 4a-4b illustrate the amino acid sequence of G α ₁₅ (SEQ. ID. NO. 16) and G α ₁₆ (SEQ. ID. NO. 17).

[0046] FIGS. 5a-5r illustrate the cDNA sequences encoding for human CaR (SEQ. ID. NO. 18), human GABA_BR1a (SEQ. ID. NO. 19), human GABA_BR1b (SEQ. ID. NO. 20), and human GABA_BR2 (SEQ. ID. NO. 21).

[0047] FIGS. 6a-6h illustrate the cDNA sequence for rat GABA_BR1a (SEQ. ID. NO. 22) and rat GABA_BR1b (SEQ. ID. NO. 23).

[0048] FIGS. 7a-7c illustrate the amino sequence for rat GABA_BR1a (SEQ. ID. NO. 24) and rat GABA_BR1b (SEQ. ID. NO. 25).

[0049] FIG. 8 illustrates the ability of a chimeric CaR/GABA_BR2 (CaR extracellular and transmembrane domains, and intracellular GABA_BR2 domain) to transduce a signal. Signal production was measured by detecting an increase in the calcium-activated chloride current. The line in the middle of the increase signifies a wash step.

[0050] FIGS. 9a-9p illustrate the cDNA sequence for human mGluR2 (SEQ. ID. NO. 26), chimeric hCAR/hmGluR2 (SEQ. ID. NO. 30), chimeric hmGluR2/hCaR (SEQ. ID. NO. 34), and chimeric hmGluR8/hCaR (SEQ. ID. NO. 38).

[0051] FIGS. 10a-10f illustrate the amino acid sequence for human mGluR2 (SEQ. ID. NO. 27), chimeric hCAR/hmGluR2 (SEQ. ID. NO. 31), chimeric hmGluR2/hCaR (SEQ. ID. NO. 35), chimeric hmGluR8/hCaR (SEQ. ID. NO. 39).

[0052] FIGS. 11a-11v illustrate the cDNA sequence for the phCaR/hmGluR2*Gqi5 fusion construct (SEQ. ID. NO. 32), pmGluR2//CaR*Gaqi5 fusion construct (SEQ. ID. NO. 36), pmGluR2//CaR*Gaqi5+3Ala linker fusion construct (SEQ. ID. NO. 46), and the mGluR8//CaR*Gaqi5 fusion construct (SEQ. ID. NO. 40).

[0053] FIGS. 12a-12h illustrate the amino acid sequence for the phCaR/hmGluR2*Gqi5 fusion construct (SEQ. ID. NO. 33), pmGluR2//CaR*Gaqi5 fusion construct (SEQ. ID. NO. 37), pmGluR2//CaR*Gaqi5+3Ala linker fusion construct (SEQ. ID. NO. 47), and the mGluR8//CaR*Gaqi5 fusion construct (SEQ. ID. NO. 41).

[0054] FIGS. 13a-13m illustrate the cDNA sequence for the GABA-R2*Gqo5 fusion construct (SEQ. ID. NO. 42) and the GABA-BR1a*Gqo5 fusion construct (SEQ. ID. NO. 44).

[0055] FIGS. 14a-14e illustrates the amino acid sequence for the GABA-BR2*Gqo5 fusion construct (SEQ. ID. NO. 43) and the GABA-BR1a*Gqo5 fusion construct (SEQ. ID. NO. 45).

[0056] FIG. 15 illustrates the ability of different G-protein fusions to transduce signals resulting from ligand binding. mGluR2//CaR*Gqi5 is shown by SEQ. ID. NO. 37, CaR/mGluR2*Gqi5 is shown by SEQ. ID. NO. 33, mGluR8//CaR*Gqi5 is shown by SEQ. ID. NO. 41.

[0057] FIGS. 16a-16e illustrates the amino acid sequence for the ph8SpmGluR4 chimeric construct (SEQ. ID. No. 48), the amino sequence for the phmGluR4/CaR*AAA*Gαqi5 fusion construct (SEQ. ID. No. 49), and the phmGluR8//CaR*AAA* Gαqi5 fusion construct (SEQ. ID. NO. 50).

DETAILED DESCRIPTION OF THE INVENTION

[0058] The CaR, mGluR, and the GABA_BR are structurally similar in that they are each a single subunit membrane protein possessing an extracellular domain, a transmembrane domain comprising seven putative membrane spanning helices connected by three intracellular and three extracellular loops, and an intracellular carboxy-terminal domain. Signal transduction is activated by the extracellular binding of an agonist. The signal is transduced to the intracellular components of the receptor causing an intracellular effect.

[0059] Signal transduction from agonist binding to an extracellular region can be modulated by compounds acting at a downstream transmembrane domain or the intracellular domain. Downstream effects include antagonist actions of compounds and allosteric actions of compounds.

[0060] The transmembrane domain provides different types of target sites for compounds modulating receptor activity in different environments. As noted above, the transmembrane domain contains extracellular, transmembrane, and intracellular components.

[0061] Compounds modulating GABA_BR, CaR, or mGluR activity can be obtained, for example, by screening a group or library of compounds to identify those compounds having the desired activity and then synthesizing such compound. Thus, included in the present invention is a method of making a GABA_BR, CaR, or mGluR active compound by first screening for a compound having desired properties and then chemically synthesizing that compound.

Metabotropic Glutamate Receptors (mGluRs)

[0062] mGluRs are G protein-coupled receptors capable of activating a variety of intracellular secondary messenger systems following the binding of glutamate (Schoepp *et al.*, *Trends Pharmacol. Sci.* 11:508, 1990; Schoepp and Conn, *Trends Pharmacol. Sci.* 14:13, 1993, hereby incorporated by reference herein).

[0063] Activation of different mGluR subtypes *in situ* elicits one or more of the following responses: activation of phospholipase C, increases in phosphoinositide (PI) hydrolysis, intracellular calcium release, activation of phospholipase D, activation or inhibition of adenylyl cyclase, increases and decreases in the formation of cyclic adenosine monophosphate (cAMP), activation of guanylyl cyclase, increases in the formation of cyclic guanosine monophosphate (cGMP), activation of phospholipase A₂, increases in arachidonic acid release, and increases or decreases in the activity of voltage- and ligand-gated ion channels (Schoepp and Conn, *Trends Pharmacol. Sci.* 14:13, 1993; Schoepp, *Neurochem. Int.* 24:439, 1994; Pin and Duvoisin, *Neuropharmacology* 34:1, 1995, hereby incorporated by reference herein).

[0064] Eight distinct mGluR subtypes have been isolated. (Nakanishi, *Neuron* 13:1031, 1994; Pin and Duvoisin, *Neuropharmacology* 34:1, 1995; Knopfel et al., *J. Med. Chem.* 38:1417; *Eur. J. Neuroscience* 7:622-629, 1995, each of these references is hereby incorporated by reference herein.) The different mGluRs possess a large amino-terminal extracellular domain (ECD) followed by seven putative transmembrane domain (7TMD) comprising seven putative membrane-spanning helices connected by three intracellular and three extracellular loops, and an intracellular carboxy-terminal domain of variable length (cytoplasmic tail).

[0065] Human mGluR8 is described by Stormann *et al.*, U.S. Patent Nos. 6,051,688, 6,077,675, and 6,084,084, International Application Number PCT/US97/09025, International Publication Number WO 97/48724, and mouse mGluR8 is described by Duvoisin *et al.*, *J. Neurosci.* 15:3075-3083, 1995 (both of these references are hereby incorporated by reference herein). mGluR8 couples to G_i. Agonists of mGluR8 include L-glutamate and L-2-amino-4-phosphonobutyrate.

[0066] mGluR8 activity can be measured using standard techniques. For example, G_i negatively couples to adenylate cyclase to inhibit intracellular cAMP accumulation in a pertussis toxin-sensitive fashion. Thus, mGluR8 activity can be measured, for example, by measuring

inhibition of forskolin-stimulated cAMP production as described by *Duvoisin et al., J. Neurosci.* 15:3075-3083, 1995.

[0067] mGluRs have been implicated in a variety of neurological pathologies. Examples of such pathologies include stroke, head trauma, spinal cord injury, epilepsy, ischemia, hypoglycemia, anoxia, and neurodegenerative diseases such as Alzheimer's disease (Schoepp and Conn, *Trends Pharmacol. Sci.* 14:13, 1993; Cunningham *et al., Life Sci.* 54:135, 1994; Pin *et al., Neuropharmacology* 34:1, 1995; Knopfel *et al., J. Med. Chem.* 38:1417, 1995, each of which is hereby incorporated by reference herein).

Calcium Receptor

[0068] The CaR responds to changes of extracellular calcium concentration and also responds to other divalent and trivalent cations. The CaR is a G-protein-coupled receptor containing an extracellular Ca^{2+} binding domain. Activation of the CaR, descriptions of CaRs isolated from different sources, and examples of CaR active compound are provided in Nemeth *NIPS* 10:1-5, 1995; Brown *et al.* U.S. Patent No. 5,688,938; Van Wagenen *et al.*, International Application Number PCT/US97/05558 International Publication Number WO 97/37967; Brown E.M. *et al., Nature* 366:575, 1993; Riccardi D., *et al., Proc. Nat'l. Acad. Sci. USA* 92:131-135, 1995; and Garrett J.E., *et al., J. Biol. Chem.* 31:12919-12925, 1995. (Each of these references are hereby incorporated by reference herein.) Brown *et al.* U.S. Patent No. 5,688,938, and Van Wagenen *et al.*, International Application Number PCT/US97/05558 International Publication Number WO 97/37967, describe different types of compounds active at the CaR including compounds that appear to be allosteric modulators and CaR antagonists.

[0069] The CaR can be targeted to achieve therapeutic effects. Examples of target diseases are provided in Brown *et al.* U.S. Patent No. 5,688,938, and Van Wagenen *et al.*, International Application Number PCT/US97/05558 International Publication Number WO 97/37967, and include hyperparathyroidism and osteoporosis.

γ -Aminobutyric acid Receptors (GABA_BRs)

[0070] GABA_BRs are G-protein-coupled metabotropic receptors. GABA_BRs modulate synaptic transmission by inhibiting presynaptic transmitter release and by increasing K^+

conductance responsible for long-lasting inhibitory postsynaptic potentials. (See, Kaupmann *et al.*, *Nature* 386:239-246, 1997, hereby incorporated by reference herein.)

[0071] GABA_BRs are found in the mammalian brain, in locations outside of the brain, and in lower species. Outside of the brain, GABA_BRs have been identified on axon terminals and ganglion cell bodies of the autonomic nervous system, on fallopian tube and uterine intestinal smooth muscle cells, in the kidney cortex, urinary bladder muscle and on testicular interstitial cells. (See, Bowery, *Annu. Rev. Pharmacol. Toxicol.* 33:109-147, 1993, hereby incorporated by reference herein.)

[0072] Different GABA_BR subtypes exist. Kaupmann *et al.*, *Nature* 386:239-246, 1997, indicate that they cloned GABA_BRs. Nucleic acid encoding two GABA_BR proteins were indicated to be cloned from rat brain: GABA_BR1a and GABA_BR1b. GABA_BR1a differs from GABA_BR1b in that the N-terminal 147 residues are replaced by 18 amino acids. GABA_BR1a and GABA_BR1b appear to be splice variants. The cloned GABA_BRs were indicated to negatively couple adenylyl cyclases and show sequence similarity to the metabotropic receptors for L-glutamate (mGluR). Northern blot analysis indicated that GABA_BR1a and GABA_BR1b are present in brain and testis, but not in kidney, skeletal muscle, liver, lung, spleen, or heart.

[0073] Kaupmann *et al.*, International Application Number PCT/EP97/01370, International Publication Number WO 97/46675, indicate that they have obtained rat GABA_BR clones, GABA_BR1a and GABA_BR1b; and human GABA_BR clones, GABA_BR1a/b (representing a partial receptor clone) and GABA_BR1b (representing a full-length receptor clone). Amino acid sequence information and encoding cDNA sequence information are provided for the different GABA_BR clones.

[0074] Another GABA_BR subtype is GABA_BR2. Northern blot analysis reveals that an approximate 6.3-Kb human GABA_BR2 transcript is abundantly expressed in the human brain. Expression is not detected in the heart, placenta, lung, liver, skeletal muscle, kidney and pancreas under conditions where GABA_BR2 transcript was identified in the human brain. Within the human brain, GABA_BR2 is broadly expressed at variable levels.

[0075] GABA_BR functions as a heterodimer of the subunits GABA_BR1 or GABA_BR2. (Jones *et al.*, *Nature* 396:674-679, 1998, hereby incorporated by reference herein.)

[0076] GABA_BRs have been targeted to achieve therapeutic effects. Kerr and Ong, DDT 1:371-380, 1996, describe different compounds indicated to be GABA_BR agonists and GABA_BR antagonists. Kerr and Ong also review therapeutic implications of affecting GABA_BR activity including, spasticity and motor control, analgesia, epilepsy, cognitive effects, psychiatric disorders, alcohol dependence and withdrawal, feeding behavior, cardiovascular and respiratory functions, and peripheral functions.

[0077] Bittiger *et al.*, *Tips* 4:391-394, 1993, review therapeutic applications of GABA_BR antagonists. Potential therapeutic applications noted by Bittiger *et al.* include cognitive processes, epilepsy, and depression.

G-Protein Fusion Receptors

[0078] Examples of some different types of G-protein fusion receptors, and advantages of some receptors, are provided below. Using the present application as a guide, additional G-protein fusion receptors can be constructed.

[0079] G-protein fusion receptors contain an intracellular domain of a receptor fused to a G-protein α subunit (G_α). G_α fusions to adrenoreceptors have been reported by Bertin *et al.*, *Receptors and Channels* 5:41-51, 1997; Wise and Milligan, *Journal of Biological Chemistry* 39:24673-24678, 1997; and Bertin *et al.*, *Proc. Natl. Acad. Sci. USA* 91:8827-8831, 1994 (each of which are hereby incorporated by reference herein). These studies were indicated to produce a functional chimeric by fusing the α_{2A} -adrenoreceptor to the $G_{i1\alpha}$, or the β_2 -adrenoreceptor to the G_{sa} .

[0080] The G-protein fusion receptors described by the present invention include a G-protein fused to an intracellular domain, where the intracellular domain, when present in a wild-type receptor, does not interact with that type of G-protein. Thus, the present invention also describes swapping of signals by fusing an intracellular domain to a G_α normally not coupled to that intracellular domain. The use of such fusion proteins, while applicable to chimeric GABA_BRs, is not limited to chimeric GABA_BRs. Indeed, such technology can be applied to receptors containing an extracellular domain, transmembrane domain and intracellular domain of a wild-type receptor.

[0081] Preferred G-protein fusion receptors contain an intracellular domain fused to a promiscuous G_α that couples to phospholipase C, resulting in the mobilization of intracellular calcium. Increases in intracellular calcium can be conveniently measured through the use of dyes. Such techniques are well known in the art and are described, for example, by Brown *et al.* U.S. Patent No. 5,688,938.

[0082] In an embodiment, G-protein fusions can also be used to decrease receptor desensitization.

[0083] Examples of promiscuous G_α s coupling to phospholipase C include naturally occurring G-proteins such as $G_{\alpha 15}$ and $G_{\alpha 16}$, and chimeric G-protein such as Gqo5 and Gqi5. Gqo5 and Gqi5 are made of a Gq portion where the five amino acids at the C-terminal are from either G_o or G_i , respectively (Conklin *et al.*, *Nature* 363:274-277, 1993, hereby incorporated by reference herein). The Gq portion of such chimeric receptors provides for phospholipase C coupling while the terminal G_o or G_i portion allows the chimeric G-protein to couple to different receptor proteins that are normally involved in inhibitor effects on adenylate cyclase.

[0084] In an embodiment of the present invention, the employed G-protein is from a human source or is made up of different G-protein components, each from a human source.

[0085] G-protein fusions can be created, for example, by fusing directly or indirectly the intracellular domain of a receptor protein to a polypeptide having an amino acid sequence substantially similar to $G_{\alpha 15}$, $G_{\alpha 16}$, Gqo5 or Gqi5. In different embodiments, the receptor is fused directly or indirectly to a G-protein consisting of the amino acid sequence of $G_{\alpha 15}$, $G_{\alpha 16}$, Gqo5 or Gqi5.

[0086] The intracellular domain portion of a receptor protein fused directly or indirectly to a G-protein should be at least about 10 amino acids in length. In different embodiments, the portion is at least about 10 amino acids, is at least about 50 amino acids, at least about 100 amino acids, or the full length of an intracellular domain.

[0087] The intracellular domain can be directly linked to a G-protein or can be indirectly linked through an optionally present linker. Optionally present linkers are preferably about 3 to about 30 amino acids in length. Preferred linkers are made up of alanine, glycine, or a combination thereof.

Chimeric Receptors

[0088] Examples of some different types of chimeric receptors, and advantages of some receptors, are provided below. Using the present application as a guide, additional chimeric receptors can be constructed.

Chimeric GABA_BR Extracellular Domain

[0089] Chimeric GABA_BRs containing a GABA_BR extracellular domain are particularly useful for studying the importance of the GABA_BR extracellular domain and assaying for compounds active at the extracellular domain. Preferably, chimeric GABA_BRs containing a GABA_BR extracellular domain also contain a CaR intracellular domain.

[0090] A variety of different activities have been generally attributed to GABA_BR subtypes. (*E.g.*, Kerr and Ong, DDT 1:371-380, 1996.) Kaupmann *et al.*, *Nature* 386:239-246, 1997, report that in preliminary experiments involving GABA_BR1a, they did not detect positive coupling to the adenylyl cyclase or coupling to the phospholipase effector system.

[0091] An intracellular CaR domain can be used to couple with G-proteins that activate phospholipase C and mobilize intracellular calcium. Mobilization of intracellular calcium is readily detected, for example, by fluorescent indicators of intracellular Ca²⁺.

[0092] An additional advantage of using the intracellular CaR domain is that CaR G-protein activation is not rapidly desensitized. Thus, the intracellular CaR domain can be used to produce a stronger intracellular signal than a signal produced from a receptor that is desensitized rapidly.

[0093] More preferably, the chimeric GABA_BR contains an intracellular CaR domain, and also contains either a CaR or a GABA_BR transmembrane domain. Advantages of using a CaR transmembrane domain include separating the effects occurring at a GABA_BR extracellular domain from effects occurring at a transmembrane domain and providing additional intracellular elements, present on transmembrane intracellular loops, useful for coupling to G-protein.

[0094] A GABA_BR transmembrane domain is useful for examining whether the transmembrane GABA_BR can be targeted to affect GABA_BR activity and obtaining compounds active at the GABA_BR transmembrane domain. For example, a transmembrane GABA_BR can be used to screen for transmembrane allosteric modulators and antagonists.

Chimeric GABA_BR Transmembrane Domain

[0095] Chimeric GABA_BRs containing a GABA_BR transmembrane are particularly useful for studying the importance of the GABA_BR transmembrane domain and assaying for compounds active at the transmembrane domain. Preferably, chimeric GABA_BRs containing a GABA_BR transmembrane domain contain an extracellular domain that is either mGluR8 or CaR, and an intracellular CaR domain.

[0096] More preferably, the chimeric GABA_BR contains an extracellular domain from either mGluR8 or CaR, a GABA_BR transmembrane, and an intracellular CaR domain. A chimeric GABA_BR containing extracellular mGluR8 or CaR domains can readily be stimulated using mGluR8 or CaR ligands.

Chimeric GABA_BR Intracellular Domain

[0097] Chimeric GABA_BRs containing a GABA_BR intracellular domain are particularly useful for studying the importance of the GABA_BR intracellular domain and assaying for compounds active at the intracellular domain. Preferably, the chimeric receptors contain an extracellular domain from either mGluR8 or CaR. The extracellular mGluR8 or CaR domains can readily be activated using mGluR8 or CaR ligands.

Receptor Domains

[0098] Domains of a G-protein fusion receptor, a chimeric receptor, and G_α, substantially similar to a particular sequence can be readily produced using the disclosure provided herein in conjunction with information well known in the art. Substantially similar sequences can be obtained taking into account sequence information for a particular type of receptor obtained from different sources, different types of amino acids that are to some extent interchangeable, and the ease of experimentation with which functional receptor activity can be assayed.

[0099] Substantially similar sequences include amino acid alterations such as deletions, substitutions, additions, and amino acid modifications. A “deletion” refers to the absence of one or more amino acid residue(s) in the related polypeptide. An “addition” refers to the presence of

one or more amino acid residue(s) in the related polypeptide. Additions and deletions to a polypeptide may be at the amino terminus, the carboxy terminus, and/or internal terminus. Amino acid “modification” refers to the alteration of a naturally occurring amino acid to produce a non-naturally occurring amino acid. A “substitution” refers to the replacement of one or more amino acid residue(s) by another amino acid residue(s) in the polypeptide. Derivatives can contain different combinations of alterations including more than one alteration and different types of alterations.

[00100] The sequences of polypeptides can be compared from different sources to help identify variable amino acids not essential for receptor activity. For example, FIG. 7 provides the rat GABA_BR1a and GABA_BR1b amino acid sequences. The rat GABA_BR1a and GABA_BR1b amino acid sequences can be compared with the human GABA_BR1a and GABA_BR1b sequences to identify conserved and variable amino acids. Derivatives can then be produced where a variable amino acid is changed, and receptor activity can be readily tested.

[00101] Similarly, the amino acid sequences for CaR, mGluR8, and G-proteins from different sources are either known in the art or can readily be obtained. Examples of such references are provided above.

[00102] While the effect of an amino acid change varies depending upon factors such as phosphorylation, glycosylation, intra-chain linkages, tertiary structure, and the role of the amino acid in the active site or a possible allosteric site, it is generally preferred that a substituted amino acid is from the same group as the amino acid being replaced. To some extent, the following groups contain amino acids that are interchangeable: the basic amino acids lysine, arginine, and histidine; the acidic amino acids aspartic and glutamic acids; the neutral polar amino acids serine, threonine, cysteine, glutamine, asparagine and, to a lesser extent, methionine; the nonpolar aliphatic amino acids glycine, alanine, valine, isoleucine, and leucine (however, because of size, glycine and alanine are more closely related and valine, isoleucine and leucine are more closely related); and the aromatic amino acids phenylalanine, tryptophan, and tyrosine. In addition, although classified in different categories, alanine, glycine, and serine seem to be interchangeable to some extent, and cysteine additionally fits into this group, or may be classified with the polar neutral amino acids.

[00103] While proline is a nonpolar neutral amino acid, its replacement represents difficulties because of its effects on conformation. Thus, substitutions by or for proline are not preferred, except when the same or similar conformational results can be obtained. The conformation conferring properties of proline residues may be obtained if one or more of these is substituted by hydroxyproline (Hyp).

[00104] Examples of modified amino acids include the following: altered neutral nonpolar amino acids such as ω -amino acids of the formula $H_2N(CH_2)_nCOOH$ where n is 2-6, sarcosine (Sar), t-butylalanine (t-BuAla), t-butylglycine (t-BuGly), N-methyl isoleucine (N-Melle), and norleucine (Nleu); altered neutral aromatic amino acids such as phenylglycine; altered polar, but neutral amino acids such as citrulline (Cit) and methionine sulfoxide (MSO); altered neutral and nonpolar amino acids such as cyclohexyl alanine (Cha); altered acidic amino acids such as cysteic acid (Cya); and altered basic amino acids such as ornithine (Orn).

[00105] Preferred derivatives have one or more amino acid alteration(s) that do not significantly affect the receptor activity of the related receptor protein. In regions of receptor domains not necessary for receptor activity, amino acids may be deleted, added or substituted with less risk of affecting activity. In regions required for receptor activity, amino acid alterations are less preferred as there is a greater risk of affecting receptor activity.

[00106] Derivatives can be produced using standard chemical techniques and recombinant nucleic acid techniques. Modifications to a specific polypeptide may be deliberate, as through site-directed mutagenesis and amino acid substitution during solid-phase synthesis, or may be accidental such as through mutations in hosts that produce the polypeptide. Polypeptides including derivatives can be obtained using standard techniques such as those described by Sambrook *et al.*, *Molecular Cloning*, Cold Spring Harbor Laboratory Press (1989). For example, Chapter 15 of Sambrook describes procedures for site-directed mutagenesis of cloned DNA.

Receptor Nucleic Acid

[00107] G-protein fusion and chimeric receptor nucleic acid can be produced based on the information provided herein along with standard recombinant nucleic acid techniques. Examples of references describing recombinant nucleic acid techniques include *Molecular*

Cloning, Sambrook *et al.*, Cold Spring Harbor Laboratory Press (1989); and *Current Protocols in Molecular Biology*, Frederick *et al.*, John Wiley & Sons, Inc. (1995).

[00108] Due to the degeneracy of the genetic code, different nucleic acid sequences can encode for a particular polypeptide. Thus, a large number of nucleic acids encoding for a receptor having the same amino acid sequence can be produced.

[00109] An embodiment of the present invention uses human nucleic acid encoding for the domains from CaR, GABA_BR1A, GABA_BR1B, GABA_BR2 and/or mGluR8. The amino acid sequences of different domains is provided in FIGS. 1-3.

Recombinant Cells

[00110] Nucleic acid expressing a functional G-Protein fusion or a chimeric receptor can be used to create transfected cell lines expressing such receptors. Such cell lines have a variety of uses such as being used for high-throughput screening for compounds modulating receptor activity, being used to assay binding to the receptor, and as factories to produce large amounts of a receptor.

[00111] A variety of cell lines can couple exogenously expressed receptors to endogenous functional responses. Cell lines such as NIH-3T3, HeLa, NG115, CHO, HEK 293 and COS7, which are expected to lack CaR, mGluR8, and GABA_BR, can be tested to confirm that they lack these receptors.

[00112] Production of stable transfectants can be accomplished by transfection of an appropriate cell line with, for example, an expression vector such as pMSG vector, in which the coding sequence for the G-protein fusion or chimeric GABA_BR cDNA has been cloned. Expression vectors containing a promoter region, such as the mouse mammary tumor virus promoter (MMTV), drive high-level transcription of cDNAs in a variety of mammalian cells. In addition, these vectors contain genes for selecting cells stably expressing cDNA of interest. The selectable marker in the pMSG vectors encode an enzyme, xanthine-guanine phosphoribosyl transferase (XGPRT), conferring resistance to a metabolic inhibitor that is added to the culture to kill nontransfected cells.

[00113] The most effective method for transfection of eukaryotic cell lines with plasmid DNA varies with the given cell type. The expression construct will be introduced into cultured

cells by the appropriate technique, such as Ca^{2+} phosphate precipitation, DEAE-dextran transfection, lipofection or electroporation. Expression of the receptor cDNA in cell lines can be assessed by solution hybridization and Northern blot analysis.

Binding Assays

[00114] The present invention also includes using G-protein fusion receptors or chimeric GABA_BR in a binding assay. G-protein fusion receptors or chimeric GABA_BRs having a particular GABA_BR domain can be used, for example, to facilitate obtaining compounds able to bind to that particular receptor domain and to determine whether a compound binds to a particular domain. For example, in a complete chimeric GABA_BR containing extracellular, transmembrane, and intracellular domains, the presence of one or more domains from CaR or mGluR are useful to present GABA_BR domain(s) to a binding agent in a form more like the GABA_BR domain(s) in the wild-type receptor, compared to an incomplete GABA_BR receptor fragment lacking one or more domains.

[00115] Binding assays can be carried out using techniques well known in the art. Binding assays preferably employ radiolabeled binding agents.

[00116] An example of a binding procedure is carried out by first attaching chimeric GABA_BR to a solid-phase support to create an affinity matrix. The affinity matrix is then contacted with potential GABA_BR binding agents. A large library of compounds may be used to determine those compounds binding to the affinity matrix. Bound compounds can be eluted from the column.

Therapeutic Modulation

[00117] As pointed out above, different types of diseases and disorders can be treated using compounds modulating CaR, mGluR, or GABA_BR activity. Additionally, such compounds can be used prophylactically. Compounds modulating GABA_BR2 activity can be administered to patients who would benefit from such treatment. Patients are mammals, preferably humans.

[00118] Modulators of CaR, mGluR, or GABA_BR activity can be administered to a patient using standard techniques. Techniques and formulations generally may be found in

Remington's Pharmaceutical Sciences 18th ed., Mack Publishing Co., Easton, PA, 1990 (hereby incorporated by reference herein).

[00119] Suitable dosage forms, in part, depend upon the use or the route of entry, for example, oral, transdermal, transmucosal, or by injection (parenteral). Such dosage forms should allow the therapeutic agent to reach a target cell whether the target cell is present in a multicellular host or in culture. For example, pharmacological compounds or compositions injected into the blood stream should be soluble. Other factors are well known in the art, and include considerations such as toxicity and dosage forms that retard the therapeutic agent from exerting its effect.

[00120] Therapeutic compounds can be formulated as pharmaceutically acceptable salts and complexes thereof. Pharmaceutically acceptable salts are non-toxic salts in the amounts and concentrations at which they are administered. The preparation of such salts can facilitate the pharmacological use by altering the physical characteristics of the compound without preventing it from exerting its physiological effect. Useful alterations in physical properties include lowering the melting point to facilitate transmucosal administration and increasing the solubility to facilitate administering higher concentrations of the drug.

[00121] The pharmaceutically acceptable salt of a compound may be present as a complex. Examples of complexes include an 8-chlorotheophylline complex (analogous to, *e.g.*, dimenhydrinate:diphenhydramine 8-chlorotheophylline (1:1) complex; Dramamine) and various cyclodextrin inclusion complexes.

[00122] Pharmaceutically acceptable salts include acid addition salts such as those containing sulfate, hydrochloride, fumarate, maleate, phosphate, sulfamate, acetate, citrate, lactate, tartrate, methanesulfonate, ethanesulfonate, benzenesulfonate, *p*-toluene-sulfonate, cyclohexylsulfamate and quinate.

[00123] Pharmaceutically acceptable salts can be obtained from acids such as hydrochloric acid, maleic acid, sulfuric acid, phosphoric acid, sulfamic acid, acetic acid, citric acid, lactic acid, tartaric acid, malonic acid, methanesulfonic acid, ethanesulfonic acid, benzenesulfonic acid, *p*-toluenesulfonic acid, cyclohexylsulfamic acid, fumaric acid, and quinic acid.

[00124] Pharmaceutically acceptable salts also include basic addition salts such as those containing benzathine, chlorprocaine, choline, diethanolamine, ethylenediamine, meglumine, procaine, aluminum, calcium, lithium, magnesium, potassium, sodium, ammonium, alkylamine, and zinc, when acidic functional groups, such as carboxylic acid or phenol are present. For example, see *Remington's Pharmaceutical Sciences* 18th ed., Mack Publishing Co., Easton, PA, p. 1445, 1990. Such salts can be prepared using the appropriate corresponding bases.

[00125] Carriers or excipients can also be used to facilitate administration of therapeutic agents. Examples of carriers include calcium carbonate, calcium phosphate, various sugars such as lactose, glucose, or sucrose, or types of starch, cellulose derivatives, gelatin, vegetable oils, polyethylene glycols and physiologically compatible solvents. Examples of physiologically compatible solvents include sterile solutions of water for injection (WFI), saline solution and dextrose.

[00126] GABA_BR-modulating compounds can be administered by different routes including intravenous, intraperitoneal, subcutaneous, intramuscular, oral, topical (transdermal), or transmucosal administration. For systemic administration, oral administration is preferred. For oral administration, for example, the compounds can be formulated into conventional oral dosage forms such as capsules, tablets, and liquid preparations such as syrups, elixirs, and concentrated drops.

[00127] Alternatively, injection (parenteral administration) may be used, *e.g.*, intramuscular, intravenous, intraperitoneal, and subcutaneous. For injection, compounds are formulated in liquid solutions, preferably, in physiologically compatible buffers or solutions, such as saline solution, Hank's solution, or Ringer's solution. In addition, the compounds may be formulated in solid form and redissolved or suspended immediately prior to use. Lyophilized forms can also be produced.

[00128] Systemic administration can be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are well known in the art, and include, for example, for transmucosal administration, bile salts and fusidic acid derivatives. In addition, detergents may be used to facilitate permeation. Transmucosal administration, for example, may be through nasal sprays, rectal suppositories, or vaginal suppositories.

[00129] For topical administration, compounds can be formulated into ointments, salves, gels, or creams, as is well known in the art.

[00130] The amounts of various GABA_BR-modulating compounds to be administered can be determined by standard procedures taking into account factors such as the compound IC₅₀, EC₅₀, the biological half-life of the compound, the age, size and weight of the patient, and the disease or disorder associated with the patient. The importance of these and other factors to be considered are well known to those of ordinary skill in the art. Generally, the amount is expected to preferably be between about 0.01 and 50 mg/kg of the animal to be treated.

EXAMPLES

[00131] Examples are provided below illustrating different aspects and embodiments of the present invention. The examples include techniques that can be used to produce and use G-protein fusion receptors and chimeric receptors. These examples are not intended to limit the claimed invention.

Example 1: Construction of G-Protein Fusions

[00132] This example illustrates different G-protein fusion receptor constructs and techniques used to produce different G-protein fusion receptor constructs. Numbering of nucleotide position for all the following constructs is such that nucleotide number 1 corresponds to the A of the ATG start codon of the nucleotide sequence encoding the designated protein.

I. FULL-LENGTH CONSTRUCTS

A. phCaR

[00133] The cDNA encoding the human CaR (Garrett *et al.*, (1995) *J. Biol. Chem.* 270:12919) is harbored in the Bluescript SK(-) plasmid (Stratagene). This construct is referred to as phCaR.

B. phmGluR2

[00134] A full-length human mGluR2 cDNA was amplified from human cerebellum MarathonReady cDNA (Clontech) using PCR primers based on the human mGluR2 cDNA

sequence (Genbank Accession # 4504136). The obtained PCR fragment was subcloned into the pT7Blue TA vector (Novagen). A Hind III-Not I fragment containing the human mGluR2 cDNA was then subcloned into the Bluescript SKII(-) plasmid (Stratagene). This construct is referred to as phmGluR2.

C. phGα_q

[00135] A full-length human Gα_q cDNA was amplified from human cerebral cortex Quick-Clone cDNA (Clontech) using PCR primers based on the human Gα_q cDNA sequence (Genbank Accession # 4504044). The obtained PCR fragment was subcloned into the Bluescript SKII(-) plasmid (Stratagene). This construct is referred to as phGα_q.

D. phmGluR8

[00136] The cDNA encoding the full-length human mGluR8 cDNA (Stormann *et al.*, U.S. Patent Nos. 6,051,688, 6,077,675, and 6,084,084, and International Publication No. WO97/48724) is harbored in the Bluescript SKII(-) plasmid (Stratagene). This construct is referred to as phmGluR8.

E. ph8SpmGluR4

[00137] A full-length human mGluR4 cDNA was amplified from human cerebellum MarathonReady cDNA (Clontech) using PCR primers based on the human mGluR4 cDNA sequence (Genbank Accession #X80818). The obtained PCR fragment was cloned into the pT7Blue TA vector (Novagen). A 2977 bp BamHI fragment containing the human mGluR4 cDNA was then subcloned into the vector pcDNA3.1/Hygro+ (Invitrogen). This construct is referred to as phmGluR4.

[00138] Next, the predicted signal peptide of mGluR4 was replaced with the predicted signal peptide and 87 bp of 5' UTR from phmGluR8 using a recombinant PCR strategy similar to those described above. The first reaction used a phmGluR8 construct with two primers, 3.1-535F (sense 21-mer, complementary to vector sequence upstream of the hmGluR8 insert; sequence 5'-ggcattatgccagttacatga-3') (SEQ ID NO:51), and the hybrid primer 8/4RP (antisense 42-mer, containing 21 nucleotides complementary to human mGluR8 and 21 nucleotides

complementary human mGluR4; sequence 5'-caagcctctcttcccaggcattttctccacaggtggtattgc-3') (SEQ ID NO:52). These primers were used to amplify a 469 bp PCR fragment of human mGluR8.

[00139] In a separate PCR reaction using phmGluR4 as template, a 472 bp fragment of human mGluR4 was amplified using a hybrid primer 4/8RP (sense 42-mer, exactly complementary to primer 8/4RP) and oligo mG4-472R (antisense 18-mer, complementary to the human mGluR4 cDNA; sequence 5'-ctgaagcaccgatgacae-3') (SEQ ID NO:53). The two PCR products generated from the above two reactions were annealed together in equimolar ratios in the presence of the external primers mG4-472R and 3.1-535F, and Turbo Pfu DNA polymerase (Stratagene).

[00140] The resulting chimeric PCR product was digested with NarI and NheI (New England Biolabs) and subcloned into phmGluR4 digested with the same two restriction enzymes. The sequence of the resultant chimeric construct, ph8SPmGluR4, was verified by ABI automated DNA sequence analysis.

[00141] The replacement of the predicted signal peptide of mGluR4 with that of mGluR8 greatly increased the activity of this receptor in *in vitro* assays.

II. $G\alpha_{q5}$

[00142] The cDNA encoding the human $G\alpha_{q5}$ cDNA (Conklin *et al.* (1993) *Nature* 363:274-77) is harbored in the Bluescript SKII(-) plasmid (Stratagene). This construct is referred to as $G\alpha_{q5}$. The nucleic acid and amino acid sequences for $G\alpha_{q5}$ are provided by SEQ. ID. NOs. 28 and 29, respectively.

III. phCaR/hmGluR2

[00143] This chimera contains the extracellular domain of the human CaR and transmembrane domain and intracellular cytoplasmic tail of human mGluR2. The chimeric junction between the CaR and hmGluR2 was created using a recombinant PCR strategy similar to those described above.

[00144] The first reaction used two primers, CA1156 (sense 19-mer, corresponding to nucleotides 1156-1174 of human CaR), and the hybrid primer CA/2 (antisense 42-mer,

containing 21 nucleotides complementary to nucleotides 1774-1794 of human CaR and 21 nucleotides complementary to nucleotides 1660-1680 of the human mGluR2). These primers were used to amplify a 659 bp PCR fragment of human CaR.

[00145] In a separate PCR reaction using phmGluR2 as template, a 692 bp fragment of the human mGluR2 was amplified using a hybrid primer 2/CA (sense 42-mer, exactly complementary to primer CA/2) and oligo 2-2330m, (antisense 23-mer, complementary to nucleotides 2309-2331 of the human mGluR2 cDNA). The two PCR products generated from the above two reactions were annealed together in equimolar ratios in the presence of the external primers CA1156 and 2-2330m, and the Pfu DNA polymerase (Stratagene).

[00146] The resulting chimeric PCR product was digested with SexA1 (Boehringer Mannheim) and BamHI (New England Biolabs) and subcloned into pHCaR digested with the same two restriction enzymes. In the final cloning step, the 3' end of human mGluR2 was subcloned into this construct using the restriction enzymes BsrGI and BamHI (both New England Biolabs). The sequence of the resultant chimeric construct, pHCaR/hmGluR2, was verified by ABI automated DNA sequence analysis.

IV. pHCaR/hmGluR2*Gqi5

[00147] This construct contains the pHCaR/hmGluR2 chimeric receptor fused to human G α qi5. A HindIII-BamHI fragment containing the pHCaR/hmGluR2 construct was subcloned into pcDNA3.1/Hygro(+) (Invitrogen) to aid in constructing this fusion protein. The chimeric junction between the C-terminus of pHCaR/hmGluR2 and the N-terminus of G α qi5 was created using a recombinant PCR strategy similar to those described above.

[00148] The first reaction used two primers, 2-1713 (sense 21-mer, corresponding to nucleotides 1710-1730 of human mGluR2) and the hybrid primer 2/Q (antisense 42-mer, containing 21 nucleotides complementary to nucleotides 2596-2616 of human mGluR2, and 21 nucleotides complementary to nucleotides 1-21 of pG α qi5). These primers were used to amplify a 927 bp PCR fragment of pHCaR/hmGluR2. In a separate PCR reaction, all of G α qi5 was amplified using a hybrid primer Q/2 (sense 42-mer, exactly complementary to primer 2/Q) and the T3 primer commercially available from Stratagene.

[00149] These two PCR products generated from the above two reactions were annealed together in equimolar ratios in the presence of the external primers 2-1713 and T3, and the Pfu DNA polymerase (Stratagene). The resulting chimeric PCR product was digested with Bsu361 and BamHI (New England Biolabs) and subcloned into phCaR/hmGluR2 digested with the same two restriction enzymes. The sequence of the resultant chimeric fusion construct, phCaR/hmGluR2*Ga_qi5, was verified by DNA sequence analysis.

V. phmGluR2//CaR Construct

[00150] This chimera contains the extracellular and transmembrane domains of human mGluR2 linked to the intracellular cytoplasmic tail domain of the human CaR. The chimeric junction was created using three separate PCR reactions.

[00151] The first reaction used two primers, 2-1713 (sense 21-mer, corresponding to nucleotides 1710-1730 of human mGluR2, Genbank Accession # 4504136) and the hybrid primer 2/CT (antisense 42-mer, containing 21 nucleotides complementary to nucleotides 2452-2472 of human mGluR2 and 21 nucleotides complementary to nucleotides 2602-2622 of the human CaR). These primers were used to amplify a 783 bp PCR fragment of human mGluR2. In a separate PCR reaction using phCaR in the BlueScript SK⁻ plasmid as template, a 750 bp fragment of the human CaR was amplified using a hybrid primer CT/2 (sense 42-mer, exactly complementary to primer 2/CT) and the T3 primer commercially available from Stratagene.

[00152] The two PCR products generated from the above two reactions were annealed together in equimolar ratios in the presence of the external primers 2-1713 and T3, and the Pfu DNA polymerase (Stratagene). The resulting chimeric PCR product was digested with BsrGI and NotI (New England Biolabs) and subcloned into pmGluR2 digested with the same two restriction enzymes. The sequence of the resultant chimeric construct, pmGluR2//CaR, was verified by ABI automated DNA sequence analysis.

VI. pmGluR2//CaR*Ga_qi5 Construct

[00153] This construct contains the hmGluR2//CaR chimeric receptor fused to human Ga_qi5. The chimeric junction between the C-terminus of hmGluR2//CaR and the N-terminus of

Gα_qi5 was created using a recombinant PCR strategy similar to that described above for the construction of phmGluR2//CaR.

[00154] The first reaction used two primers, CRP10A (sense 18-mer, corresponding to nucleotides 2812-2829 of phCaR) and the hybrid primer CaRQ (antisense 42-mer, containing 21 nucleotides complementary to nucleotides 3214-3234 phCaR, and 21 nucleotides complementary to nucleotides 1-21 of pGα_qi5). These primers were used to amplify a 443 bp PCR fragment of hmGluR2//CaR. In a separate PCR reaction, all of Gα_qi5 was amplified using a hybrid primer QCaR (sense 42-mer, exactly complementary to primer CaRQ) and the T3 primer commercially available from Stratagene.

[00155] The two PCR products generated from the above two reactions were annealed together in equimolar ratios in the presence of the external primers CRP10A and T3, and the Pfu DNA polymerase (Stratagene). The resulting chimeric PCR product was digested with BstEII and NotI (New England Biolabs) and subcloned into pmGluR2//CaR digested with the same two restriction enzymes. The sequence of the resultant chimeric fusion construct, pmGluR2//CaR*Gα_qi5, was verified by ABI automated DNA sequence analysis.

VII. Fusion Receptor Protein Linker Addition Constructs

A. phmGluR2//CaR*AAA*Gα_qi5

[00156] A linker encoding three alanine residues was incorporated into the phmGluR2//CaR*Gα_qi5 construct by mutagenesis (Stratagene QuickChange Mutagenesis Kit). A sense 40-mer, 2CQ+LP, contained 16 nucleotides corresponding to 3219-3234 of human CaR, followed by the nine nucleotide sequence (GCGGCCGCC) encoding three alanine residues and a NotI restriction enzyme site, and then 15 nucleotides corresponding to nucleotides 1-15 of Gα_qi5. 2CQ+LP was annealed to an antisense 40-mer, 2CQ+LM, the exact complement of 2CQ+LP. These oligos were used in the mutagenesis reaction according to the manufacturer's protocol. Restriction enzyme analysis and DNA sequence analysis confirmed the insertion of the nine nucleotide linker (GCGGCCGCC) between the C-terminus of phmGluR2//CaR and the N-terminus of Gα_qi5. This construct was designated phmGluR2//CaR*AAA*Gα_qi5.

B. Human GABA_BR2*AAA*Gα_qo5 and human GABA_BR1a*AAA*Gα_qo5

[00157] These constructs contain the human GABA_BR2 (hGABA_BR2: Genbank Accession # AJ 012188) and human GABA_BR1a (hGABA_BR1a: Genbank Accession # AJ 012185) fused at their C-terminus to the N-terminus of human Gα_qo5 (hGα_qo5: *Nature* 363:274-276, 1993). Human GABA_BR2, hGABA_BR1a, and hGα_qo5 were cloned into the plasmid pcDNA3.1/Hygro+ (Invitrogen) and are designated phGABA_BR2, phGABA_BR1a, and phGα_qo5. The first reaction used two primers, XcmI-R2 (sense 20-mer, corresponding to nucleotides 2650-2669 of phGABA_BR2) and the hybrid primer R2/Go5(-) (antisense 45-mer, containing 18 nucleotides complementary to nucleotides 2806-2823 of phGABA_BR2 and 18 nucleotides complementary to nucleotides 1-18 of hGα_qo5). These two complementary areas flank a nine nucleotide sequence coding for three alanine sequences with a unique NotI restriction site. These primers were used to amplify a 200 base-pair PCR fragment.

[00158] In a separate PCR reaction, part of hGα_qo5 was amplified using a hybrid primer R2/Gα_qo5(+) (sense 45-mer), exactly complementary to R2/Go5(-) and XbaI-Go5 primer (22-mer containing 22 nucleotides complementary to nucleotides 873-895 of hGα_qo5). These primers were used to amplify a 914 base-pair PCR product. The two PCR products generated from the above two reactions were annealed together in equimolar ratios in the presence of the external primers, XcmI-R2 and XbaI-Go5, and Pfu polymerase (Stratagene).

[00159] The resulting chimeric PCR product was digested with the restriction endonucleases XcmI and XbaI (New England Biolabs) and subcloned into phGABA_BR2 digested with the same two restriction enzymes. The resulting clone was then digested with HindIII and XbaI and subcloned into phGα_qo5 cut with HindIII and XbaI resulting in the chimeric hGABA_BR*AAA*Gα_qo5. The chimeric junction between the C-terminus hGABA_BR1a, the Ala linker, and the N-terminus of hGα_qo5 was created using a recombinant PCR strategy similar to those described above.

[00160] To construct hGABA_BR1a*AAA*Gqo5, the first reaction used a commercially available T7 primer (Novagen) and the NtI hGBR1 primer (CAGAGTCATGGCGGCCCTTATAAAGCAAATGCACTCG) (SEQ ID NO:54) corresponding to nucleotide numbers 1-9 of hGα_qo5 and nucleotide numbers 2863-2883 of hGABA_BR1a.

C. phmGluR8//CaR*AAA*G α _qi5

[00161] A linker encoding three alanine residues was incorporated into the phmGluR8//CaR*G α _qi5 construct by mutagenesis (Stratagene QuickChange Mutagenesis Kit), exactly as described in Section A above, to create phmGluR2//CaR*AAA*G α _qi5. The same primers, 2CQ+LP and 2CQ+LM, were used for this mutagenesis. Restriction enzyme analysis and DNA sequence analysis confirmed the insertion of the nine-nucleotide linker (GCGGCCGCC) between the C-terminus of phmGluR8//CaR and the N-terminus of G α _qi5. This construct was designated phmGluR8//CaR*AAA*G α _qi5.

D. ph8SPmGluR4//CaR*AAA*G α _qi5

[00162] This chimera contains the extracellular and transmembrane domains of the human 8SPmGluR4 construct and intracellular cytoplasmic tail of human CaR fused to G α _qi5 through the three-alanine residue linker.

[00163] The chimeric junction between the human 8SPmGluR4 and hCaR was created using a recombinant PCR strategy similar to those previously described. The first reaction used two primers, mG4-2028R (sense 19-mer, corresponding to nucleotides of human 8SPmGluR4; sequence 5'-catctaccgcatcttcgag-3') (SEQ ID NO:55), and the hybrid primer 4CT (antisense 42-mer, containing 21 nucleotides complementary to human 8SPmGluR4 and 21 nucleotides complementary to human CaR; sequence 5'-acgcacctcctcgatgggtgttctgctccgggtggaagaggat-3') (SEQ ID NO:56). These primers were used to amplify a 549 bp PCR fragment from human 8SPmGluR4.

[00164] In a separate PCR reaction, using phmGluR2//CaR*AAA*G α _qi5 as a template, a 743 bp fragment of the human CaR*AAA*G α _qi5 was amplified using the hybrid primer CT4 (sense 42-mer, exactly complementary to primer 4CT) and oligo G α qi58R (antisense 21-mer, complementary to G α _qi5 cDNA; sequence 5'-ctcgatctcgtcgttgatccg-3') (SEQ ID NO:57). The two PCR products generated from the above two reactions were annealed together in equimolar ratios in the presence of the external primers mG4-2028R and G α qi58R, and Pfu DNA polymerase (Stratagene).

[00165] The resulting chimeric PCR product was digested sequentially with KpnI and NotI (New England Biolabs) and subcloned into ph8SPmGluR4 prepared with the same two restriction enzymes. This intermediate construct was known as ph8SPmGluR4//CaR(no stop). In the final cloning step, a fragment containing the $G\alpha_{q5}$ cDNA was released from phmGluR8//CaR*AAA* $G\alpha_{q5}$ using the restriction enzymes ApaI and NotI (both New England Biolabs) and subcloned into the ph8SPmGluR4//CaR(no stop) construct, which was prepared with the same restriction enzymes. The sequences of the resultant chimeric construct, ph8SPmGluR4//CaR*AAA* $G\alpha_{q5}$, was verified by ABI automated DNA sequence analysis.

VIII. phmGluR8//CaR Construct

[00166] This chimera contains the extracellular and transmembrane domains of human mGluR8 linked to the intracellular cytoplasmic tail domain of the human CaR. The chimeric junction between hmGluR8 and the CaR was created using a recombinant PCR strategy similar to those described above.

[00167] The first reaction used two primers, CH5A (sense 19-mer, corresponding to nucleotides 2187-2205 of human mGluR8, Stormann *et al.*, U.S. Patent Nos. 6,051,688, 6,077,675, and 6,084,084, and International Publication Number WO97/48724) and the hybrid primer CH5B (antisense 40-mer, containing 22 nucleotides complementary to nucleotides 2523-2544 of human mGluR8, and 18 nucleotides complementary to nucleotides 2602-2619 of the human CaR). These primers were used to amplify a 375 bp PCR fragment of human mGluR8. In a separate PCR reaction using phCaR in the BlueScript SK(-) plasmid as template, a 750 bp fragment of the human CaR was amplified using a hybrid primer CH5C (sense 40-mer, exactly complementary to primer CH5B) and the T3 primer commercially available from Stratagene.

[00168] The two PCR products generated from the above two reactions were annealed together in equimolar ratios in the presence of the external primers CH5A and T3, and the Pfu DNA polymerase (Stratagene). The resulting chimeric PCR product was digested with BsrGI and XbaI (New England Biolabs) and subcloned into pmGluR8 digested with the same two restriction enzymes. The sequence of the resultant chimeric construct, pmGluR8//CaR, was verified by DNA sequence analysis.

IX. mGluR8//CaR*G α _qi5 Construct

[00169] This construct contains the hmGluR8//CaR chimeric receptor fused to human G α _qi5. The chimeric junction between the C-terminus of hmGluR8//CaR and the N-terminus of G α _qi5 was created using a recombinant PCR strategy similar to that described above for the construction of phmGluR2//CaR*G α _qi5.

[00170] The first reaction used two primers, CRP10A (sense 18-mer, corresponding to nucleotides 2812-2829 of phCaR) and the hybrid primer Gqi5/CaR (antisense 40-mer, containing 21 nucleotides complementary to nucleotides 3214-3234 phCaR, and 19 nucleotides complementary to nucleotides 1-19 of pG α _qi5). These primers were used to amplify a 441 bp PCR fragment of hmGluR8//CaR.

[00171] In a separate PCR reaction, all of G α _qi5 was amplified using a hybrid primer CaR/Gqi5 (sense 40-mer, exactly complementary to primer Gqi5/CaR) and the ApaI-mut primer (20-mer). The two PCR products generated from the above two reactions were annealed together in equimolar ratios in the presence of the external primers CRP10A and ApaI-mut, and the Pfu DNA polymerase (Stratagene).

[00172] The resulting chimeric PCR product was digested with BstEII and ApaI (New England Biolabs) and subcloned into pmGluR8//CaR digested with the same two restriction enzymes. The sequence of the resultant chimeric fusion construct, pmGluR8//CaR*G α _qi5, was verified by DNA sequence analysis.

Example 2: Functional Expression of CaR/GABA_BR2

[00173] *In vitro*-transcribed RNA (7 ng) encoding a chimeric CaR/GABA_BR2 (CaR extracellular and transmembrane domains, and intracellular GABA_BR2 domain) was co-injected with *in vitro*-transcribed RNA (2 ng) encoding G α ₁₅ into *Xenopus* oocytes. Following a 72-hour incubation, the oocytes were voltage-clamped using standard electrophysiological techniques (B. Hille, *Ionic Channels of Excitable Membranes*, pp.30-33, Sinauer Associates, Inc., Sunderland, Ma., 1992). Activation of the chimeric receptor was detected by increases in the calcium-activated chloride current.

[00174] Application of the CaR activator 100 μM Gd^{3+} , resulted in reversible, oscillatory increases in the calcium-activated chloride current as shown in FIG. 8. These data demonstrate the functional response of the chimeric CaR/GABA_BR2 receptor upon activation via a site within the CaR extracellular domain. In this assay, the G_α15 subunit acts to promote signal transduction through intracellular pathways that mobilize intracellular Ca^{++} .

Example 3: Expression of Different G-Protein Fusion Receptors

[00175] The ability of different G-protein fusions to transduce signals resulting from ligand binding is shown in FIG. 15. The different G-protein fusion receptors used in this example were as follows: mGluR2//CaR*Gqi5 (SEQ. ID. NO. 37), CaR/mGluR2*Gqi5 (SEQ. ID. NO. 33), and mGluR8//CaR*Gqi5 SEQ. ID. NO. 41.

[00176] Oocytes suitable for injection were obtained from adult female *Xenopus laevis* toads using procedures described in C. J. Marcus-Sekura and M. J. M. Hitchcock, *Methods in Enzymology*, Vol. 152 (1987).

[00177] Receptor fusion cRNAs were dissolved in water and 50 nl (12.5 ng/oocyte) were injected into individual oocytes. Following injection, oocytes were incubated at 16°C in MBS containing 1 mM CaCl_2 for two to seven days prior to electrophysiological recording.

[00178] Test substances were applied by superfusion at a flow rate of about 5 ml/minute. Receptor fusion activation was determined by measuring the increase in calcium-activated chloride current (I_{Cl}). Increases in I_{Cl} were quantified by measuring the peak inward current stimulated by activating agent, relative to the holding current at -60 mV. Application of 100 μM L-glutamate elicited a response from the mGluR2//CaR*G α qi5 and mGluR8//CaR*G α qi5. Application of 100 μM Gd^{3+} activated the CaR/mGluR2*Gqi5.

Example 4: Expression of Different G-Protein Fusion Receptors in Mammalian Cells

[00179] HEK293 cells were transiently transfected with the p8SPhmGluR4//CaR*AAA*G α qi5 or phmGluR8//CaR*G α qi5 plasmid DNAs using the following protocol. Initially, 150 cm^2 tissue culture flasks containing HEK293 cells at 75% confluence were transfected with 24 μg of plasmid DNA using Gibco BRL Life Technologies' Lipofectamine reagent. Following liposomal gene delivery, the cells were allowed to recover for

24 hours. They were then plated overnight at 100,000 cells per well in black, clear bottom, Collagen I-coated 96-well plates (Becton Dickinson, Biocoat) using DMEM supplemented with 10% fetal bovine serum (Hyclone Laboratories). The cells were assayed for function 48 hours after transient transfection.

[00180] On the day of the assay, tissue culture medium was aspirated from the wells of a 96-well plate and 80 μ L of Assay Buffer (Assay Buffer is: 20 mM HEPES, 146 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mg/ml BSA, 1 mg/ml glucose, pH 7.4) supplemented with 6 μ M of the Ca^{2+} -sensitive dye, Fluo-3 AM (Molecular Probes) and 0.025% Pluronic (Molecular Probes) was added to each well.

[00181] The plate was then incubated in the dark for one hour at room temperature to efficiently load the cells with Fluo-3. At the end of the incubation, extracellular Fluo-3 was removed by washing the plate with Assay Buffer. Assay Buffer was added back to each well (final volume = 160 μ L) prior to beginning the assay. The plate was loaded into a fluorescence imaging plate reader (FLIPR) robotic device (Molecular Devices) with the laser setting at 0.8 Watts. At a time of 15 seconds after initiation of the assay, 40 μ L of Assay Buffer containing 150 μ M L-AP4 was added to the 160 μ L of Assay Buffer in each well of the plate to yield a final concentration of 30 μ M L-AP4.

[00182] Relative fluorescence intensity (excitation λ = 488 nm / emission λ = 510 nm) was monitored at relevant time intervals throughout the assay period to measure L-AP4-induced receptor activation.

[00183] Other embodiments are within the following claims. Thus, while several embodiments have been shown and described, various modifications may be made, without departing from the spirit and scope of the present invention.